

SAMUEL OLIVEIRA BORGES

**DIGESTIBLE METHIONINE + CYSTEINE:DIGESTIBLE LYSINE RATIO IN DIETS
FOR BROILERS SUBMITTED TO INFLAMMATORY CHALLENGE**

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Arele Arlindo Calderano

Co-advisers: Luiz Fernando Teixeira Albino
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**Arele Arlindo
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To God and to my family.

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"If you don't risk anything, you risk everything".
(Rob Moore)

ABSTRACT

BORGES, Samuel Oliveira, M.Sc., Universidade Federal de Viçosa, July, 2023. **Digestible methionine + cysteine:digestible lysine ratio in diets for broilers submitted to inflammatory challenge**. Adviser: Arele Arlindo Calderano. Co-advisers: Luiz Fernando Teixeira Albino and Alysson Saraiva.

To estimate the ideal digestible (dig) Methionine (Met) + Cysteine (Cys):dig Lysine (Lys) ratio for broilers submitted or not to lipopolysaccharides (LPS) (*E. coli*) inflammatory challenge, 384 thirteen-day-old male chickens were distributed in a 4 x 2 (four ratios x with or without challenge) factorial completely randomized design, with eight replicates per treatment and six chickens per experimental unit (EU). The unchallenged birds received an application of saline solution (SS). The experimental diets were formulated to meet nutritional recommendations except for the dig Met + Cys levels, which varied according to the treatments. To obtain the experimental diets, a ration with a ratio of 0.69 dig Met + Cys:dig Lys was initially prepared, and later a diet with a ratio of 0.84. These rations were mixed in the appropriate proportions to obtain diets with ratios of 0.74 and 0.79. Parameters evaluated at 21 days of age included broilers' performance, specifically weight gain (WG), feed intake (FI), and feed conversion ratio (FCR). Further parameters were jejunum mRNA content of nuclear factor kappa-B (NF-Kb), glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione synthetase (GSS), methionine adenosyltransferase 2 (MAT2), cystathionine β -synthase (CBS), relative weights of liver and spleen, and dual-energy x-ray absorptiometry (DEXA) estimated fat mass (%) and lean mass (%). The differences were considered significant at an alpha level of 0.05. A linear regression model was used to evaluate the effects of the dig Met + Cys:dig Lys ratio on the evaluated parameters. There was no interaction ($P > 0.05$) between the inflammatory challenge and the increase of dig Met + Cys: dig Lys ratio for WG, FI, FCR, the relative weight of liver and spleen, and mRNA content of NF-Kb, GPX, SOD, GSS, MAT2, and CBS. The different ratios had no significant effect ($P > 0.05$) on the same data. However, there were significant differences ($P < 0.05$) between challenged and unchallenged animals. Chickens that received an intraperitoneal application of LPS all had lower performance parameters, and the liver and spleen had heavier and lower expression of GSS than those that received SS. No significant differences ($P > 0.05$)

were observed concerning DEXA estimated fat and lean mass. Thus, the lowest dig Met + Cys:dig Lys ratio, that is 0.69 (1.193% dig Lys: 0.823% dig Met + Cys), was sufficient to maintain the parameters evaluated.

Keywords: Sulfur amino acids. Poultry. LPS. *E. coli*.

RESUMO

BORGES, Samuel Oliveira, M.Sc., Universidade Federal de Viçosa, julho de 2023. **Relação ideal metionina + cisteína: lisina em dietas para frangos de corte submetidos a desafio inflamatório.** Orientador: Arele Arlindo Calderano. Coorientadores: Luiz Fernando Teixeira Albino e Alysson Saraiva.

Para estimar a relação ideal de Metionina (Met) + Cisteína (Cis) digestível (dig): Lisina (Lis) dig para frangos de corte submetidos ou não a desafio inflamatório com lipopolissacarídeos (LPS) (*E. coli*), 384 frangos de corte machos com treze dias de idade foram distribuídos em delineamento fatorial 4 x 2 (quatro relações de Met + Cis:Lis x com ou sem desafio) inteiramente casualizado, com oito repetições e seis animais por unidade experimental (UE). As aves não desafiadas receberam aplicação de solução salina (SS). As dietas experimentais foram formuladas para atender às recomendações nutricionais, exceto para os níveis de Met + Cis dig, que variaram de acordo com os tratamentos. Para a obtenção das dietas experimentais, preparou-se inicialmente uma ração com relação de 0,69 de Met + Cis dig:Lis dig e, posteriormente, outra com relação de 0,84. Essas foram misturadas nas proporções adequadas para a obtenção das dietas com proporções de 0,74 e 0,79. Os parâmetros avaliados aos 21 dias de idade foram: desempenho - Ganho de Peso (GP), Consumo de Ração (CR) e Conversão Alimentar (CA); expressão de RNAm do jejuno dos genes alvo: fator nuclear kappa-B (NF-Kb), glutathione peroxidase (GPX), superóxido dismutase (SOD), glutathione sintetase (GSS), metionina adenosiltransferase 2 (MAT2), cistationina β -sintase (CBS); peso relativo de fígado e baço, e massa gorda (%) e magra (%) estimada por absorciometria de raios X de dupla energia (DEXA). As diferenças foram consideradas significativas ao nível alfa de 0,05. Um modelo de regressão linear foi usado para avaliar os efeitos da relação Met + Cis dig:Lis dig sobre os parâmetros avaliados. Não houve interação ($P > 0,05$) entre o desafio inflamatório e o aumento da relação Met + Cis dig:Lis dig para GP, CR, CA, peso relativo do fígado e baço e expressão de RNAm de NF-Kb, GPX, SOD, GSS, MAT2 e CBS. As diferentes relações não tiveram efeito significativo ($P > 0,05$) nos mesmos dados. No entanto, houveram diferenças significativas ($P < 0,05$) entre animais desafiados e não desafiados. As aves que receberam aplicação intraperitoneal de LPS tiveram todos os parâmetros de desempenho prejudicados, o fígado e baço mais pesados e menor expressão de GSS

em relação aos que receberam aplicação de SS. Não foram observadas diferenças significativas ($P > 0,05$) em relação à gordura e massa magra estimadas pelo DEXA. Assim, a menor relação Met + Cis dig:Lis dig, ou seja 0,69 (1,193% Lis dig: 0,823% Met + Cis dig), foi suficiente para manter os parâmetros avaliados.

Palavras-chave: Aminoácidos sulfurados. Frangos de corte. LPS. *E. coli*.

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SUMMARY

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**DIGESTIBLE METHIONINE + CYSTEINE:DIGESTIBLE LYSINE RATIO IN DIETS
FOR BROILERS SUBMITTED TO INFLAMMATORY CHALLENGE**

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1. INTRODUCTION

Currently, in intensive production, broilers are subject to a chronic inflammatory challenge that triggers immune responses impairing immunological homeostasis (Zhang et al., 2019a) due to pathogenic microorganisms present in the production system, vaccinations with excessive dosages, abuse, or lack of chemotherapy (Liu et al., 2015). This challenge prevents poultry from expressing their maximum genetic and economic potential (Li et al., 2015).

In experimentation, inoculation of bacterial LPS is a technique used to increase the activation of chickens' inflammatory response without submitting them to potentially pathogenic agents while simulating a condition closer to that found in commercial sheds (Beutler & Rietschel, 2003; Nunes et al., 2020). When inoculated and perceived by the immune system, there is an increase in the production of the NF-Kb transcription factor that stimulates the expression of inflammatory genes (Liu et al., 2022).

The sulfur amino acids (SAA) Met and Cys are involved in functions that fortify broilers' immune system, including regulation of the innate immune system, lipid metabolism, digestive functions, protein synthesis, and defense against oxidative stress (OS; Martínez et al., 2017).

The leading cause of oxidative stress (OS) is the imbalance between the production of free radicals, such as reactive oxygen species (ROS), and antioxidant enzymes (Nawaz & Zhang, 2021). Adding high levels of SAA to feed increases the synthesis and activity of GPX and SOD (Wen et al., 2016), thereby decreasing the presence of free radicals that damage macromolecules.

An increase in Met transsulfuration mitigates the effects of OS and maintains Cys' demand for cellular reduced glutathione (GSH) synthesis (Del Vesco et al., 2015; Bortoluzzi et al., 2019). Moreover, SAA participates in GSS gene expression resulting in the effective synthesis of potent antioxidants that contribute to the removing system's ability of ROS to alleviate the OS effect (Lai et al., 2018; Lugata et al., 2022). Therefore, additional SAA supplementation may improve performance by reducing OS and, consequently, the inflammatory challenge. One strategy to mitigate the adverse effects of inflammation is to increase the intake of dig SAA through the ratio dig SAA:dig Lys, such as dig Met + Cys. However, not many studies in the literature estimate the ideal dig Met + Cys:dig Lys ratio in diets for broiler chickens submitted to an inflammatory challenge.

Thus, it was hypothesized that the ideal ratio of dig Met + Cys:dig Lys in broiler diets is altered in immunologically challenged broilers. So, the objective of this study was to estimate the ideal ratio of dig Met + Cys:dig Lys for broilers submitted or not to LPS inflammatory challenge.

2. MATERIALS AND METHODS

2.1 Ethics Committee

The Animal Care and Use Committee of the Federal University of Viçosa, Brazil, approved all animal procedures (protocol no. 70/2021). The experiment was conducted according to the experimental protocols for using live broilers from the Brazilian College of Animal Experimentation.

2.2 Birds and experimental design

One-day-old male broiler chickens (Cobb 500) were obtained from a commercial hatchery. The poultry was reared on the floor according to lineage management recommendations until the beginning of the experiment. They had free access to water and were fed a corn/soybean meal-based standard diet in mashed form (*ad libitum*). At thirteenth days old and based on their body weight, a total of 384 male broilers (514 ± 51.4 g) were distributed in a 4 x 2 (four ratios of dig Met + Cys:dig Lys x with or without challenge) factorial completely randomized design, with eight replicates per treatment and six chickens per experimental unit (EU). The birds were housed in 64 EUs consisting of wire floor cages (667 cm²/broiler) in a two-level battery equipped with a trough feeder and a nipple drinker. The temperature was maintained according to lineage management recommendations, and the broilers were exposed to 18 hrs of continuous light daily during the experimental period. To simulate a chronic inflammatory challenge, birds from treatments 5, 6, 7, and 8 received an intraperitoneal application of 1mg of LPS / kg of body weight at 14, 16, 18, and 20 days old. The birds of treatments 1, 2, 3, and 4 received an application of a similar amount of SS to cause the same catching stress.

2.3 Diets

The experimental diets (Table 1) were based on corn and soybean meal, formulated to attend to the nutritional recommendations by Rostagno et al. (2017) for the 8 to 21 days old phase, except for the levels of dig Met + Cys, which varied according to the treatments. The ratios of dig Met + Cys:dig Lys tested were 0.69, 0.74, 0.79, and 0.84.

A dig lysine level of 95% of that recommended was used. The suboptimal level aimed to ensure that the chickens consumed all dig Lys and that the ideal dig Met + Cys:dig Lys ratio was not underestimated. The levels of the amino acids (Aas) threonine and arginine were increased by 0.10% and 0.15%, respectively, in an attempt to prevent these Aas from limiting the response to the increase of dig Met + Cys:dig Lys ratios of the diets. Samples of experimental diets were collected for analysis (crude protein and Aas). In addition, samples of corn and soybean meal were collected for analysis of Aas and protein levels to correct the formulas before preparing the experimental diets (Table 2). All experimental diets had an essential nitrogen:total nitrogen ratio lower than 0.50 as recommended by Maia et al. (2021).

Table 1. Ingredients and nutrient composition of experimental diets, as fed basis (%).

Ingredients	Ratio 0.69	Ratio 0.74	Ratio 0.79	Ratio 0.84
Corn (%)	51.932	51.932	51.932	51.932
Soybean Meal (%)	40.641	40.641	40.641	40.641
Soybean Oil (%)	3.452	3.452	3.452	3.452
Dicalcium Phosphate (%)	1.635	1.635	1.635	1.635
Limestone (%)	0.861	0.861	0.861	0.861
Salt (%)	0.515	0.515	0.515	0.515
DL-Methionine, 990 g/kg (%)	0.217	0.275	0.334	0.398
Starch (%)	0.181	0.123	0.064	0.000
Trace Mineral Premix ² (%)	0.130	0.130	0.130	0.130
Vitamin Premix ¹ (%)	0.130	0.130	0.130	0.130
L-Threonine, 985 g/kg (%)	0.105	0.105	0.105	0.105
Choline Chloride (%)	0.100	0.100	0.100	0.100
L-Triptofano, 980 g/kg (%)	0.056	0.056	0.056	0.056
L-Lysine HCl, 780 g/kg (%)	0.041	0.041	0.041	0.041
Calculated Composition				
Metabolizable Energy (MJ/kg)	12.770	12.770	12.770	12.770
Crude Protein (%)	23.000	23.000	23.000	23.000

Calcium (%)	0.878	0.878	0.878	0.878
Available Phosphorous	0.419	0.419	0.419	0.419
Sodium (%)	0.218	0.218	0.218	0.218
Digestible Lys (%)	1.193	1.193	1.193	1.193
Digestible Met + Cys (%)	0.823	0.883	0.942	1.002
Digestible Valine (%)	0.931	0.931	0.931	0.931
Digestible Threonine (%)	0.887	0.887	0.887	0.887
Digestible Tryptophan (%)	0.215	0.215	0.215	0.215

Analyzed Composition

Crude Protein (%)	22.410	23.930	23.400	23.830
Lys (%)	1.320	1.350	1.350	1.370
Met + Cys (%)	0.620	0.740	0.870	0.890
Valine (%)	1.080	1.080	1.050	1.060
Threonine (%)	0.960	0.960	0.940	0.980
Tryptophan (%)	0.150	0.156	0.156	0.157

¹Vitamin premix provided per kg of diet: vitamin A, 9638000 UI; vitamin D3, 2410000 UI; vitamin E, 36100 UI; vitamin K3, 1936 mg; vitamin B1, 2600 mg; vitamin B2, 6450 mg; vitamin B12, 159 mg; vitamin B6, 3610 mg; vitamin B5, 12.95 g; vitamin B3, 39.2 g; vitamin B9, 903.0 mg; biotin, 89.8 mg.

²Trace mineral premix provided per kg diet: Mn, 58.36 g; Zn, 54.21 g; Fe, 41.68 g; Cu, 8.31 g; I, 843.00 mg; Se, 250.00 mg.

Table 2. Composition of corn and soybean meal, as fed basis.

Analysis	Corn	Soybean Meal
Dry Matter (%)	89.81	88.40
Crude Protein (%)	7.44	46.37
Methionine (%)	0.16	0.63
Cysteine (%)	0.11	0.70
Lysine (%)	0.24	2.87
Threonine (%)	0.26	1.88
Valine (%)	0.33	2.19
Tryptophan (%)	0.07	0.35
Hydroxyproline (%)	0.03	0.17
Phenylalanine (%)	0.32	2.34
Leucine (%)	0.77	3.43
Isoleucine (%)	0.24	2.08
Tyrosine (%)	0.24	1.71
Proline (%)	0.66	2.44
Alanine (%)	0.52	2.24
Arginine (%)	0.36	3.43
Taurine (%)	0.03	<0.01
Histidine (%)	0.19	1.18
Glycine (%)	0.32	2.23
Serine (%)	0.34	2.48
Glutamic Acid (%)	1.27	8.45
Aspartic Acid (%)	0.50	5.41
Total Amino Acids Sum (%)	6.96	46.21

2.4 Performance and sample collection

The broilers' performance was evaluated at 21 days old by determining WG, FI, and FCR. At 20 days, one bird with a weight closest to the average weight of the EU was selected for collecting samples. Then, 24 hours after the last application of LPS, the

bird was euthanized and slaughtered. 2 cm of the jejunum was collected and stored individually in cryogenic tubes and kept in an ultra-freezer until analyzed to measure mRNA content. At 21 days of age, when one bird per EU was slaughtered for sample collection, the leftover feed was weighed, and the feed intake per bird from 13 to 21 days of age was calculated. The total feed intake per bird and the final weight were used to determine the feed conversion ratio. The liver and spleen of this broiler were removed and weighed separately to determine their relative weights. The relative weights were determined in relation to the animal's live weight.

2.5 Determination of mRNA content

The total RNA of the samples from the jejunum was extracted using Trizol® reagent (Invitrogen, Carlsbad, California, 279 USA) according to the manufacturer's instructions, in the proportion of 1 mL for each 80 mg of tissue, in order to measure mRNA expression for NF-Kb, GPX, SOD, GSS, MAT2, and CBS. RNA integrity was assessed on a 1% agarose gel stained with ethidium bromide (10 mg/mL) and visualized under ultraviolet light. For real-time PCR, the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA) was used. The amplification conditions in the thermocycler were initially denatured at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 min. The melting curves were performed to guarantee the specificity of the PCR products. The β -actin gene was used as an endogenous control (Table 3).

Table 3. Primer sequences for quantitative reverse transcription-PCR.

Gene	Primer sequence (5' to 3')
NF-Kb	F:GTGTGAAGAAACGGGAACTG R:GGCACGGTTGTCATAGATGG
GPX	F: GACCAACCCGCAGTACATCA R: GAGGTGCGGGCTTTCCTTTA
SOD	F:AGGGGGTTCATCCACTTCC R:CCCATTTGTGTTGTCTCAA
GSS	F:GTGCCAGTTCCAGTTTTCTTATG R:TCCCACAGTAAAGCCAAGAG
MAT2	F:CTTCCCAGCAGCCACTTGAG R:GCAGTCAAGCTGAGCGTTCC

CBS	F:CTGGGATCTTGAAACCTGGA R:TCAGGACATCCACCTTCTCC
β -ACTIN	F:ATTGTCCACCGCAAATGCTTC R:AAATAAAGCCATGCCAATCTCGTC

2.6 DEXA estimated fat mass (%) and lean mass (%)

Chicken body composition was measured using the Prodigy Advance DEXA scanner (GE Medical Systems Ultrasound & Primary Care Diagnostics LLC 3030 Ohmeda Drive, Madison, WI, 53718, USA) with encore software version 18. A complete body scan was performed and analyzed in the small animal body mode. At the start of each scanning day, a quality assurance program was performed using a phantom standard to ensure accurate calibration of the scanner. Eight euthanized chickens per treatment were placed in a dorsal position with spread wings and stretched legs to avoid extensive body parts overlap. Afterward, the lines defining the regions of interest were corrected for the appropriate body. Since these lines are fixed at specific intersections, a calculated compromise was consistently applied. Based on the attenuation of the 2 X-ray beams by different absorbing materials, the software calculated the estimated values for total tissue, lean and fat tissue. These values were obtained for the whole body region. After, equations developed by Schallier et al. (2019) were used to correct the values of total body fat and lean percentage (%):

Total Body Fat Percentage (%) = $-1.288(\pm 2.597) + 0.806(\pm 0.159) \times \text{Fat Percentage DEXA} (\%)$

Total Body Lean percentage (%) = $19.95(\pm 13.47) + 0.805(\pm 0.163) \times \text{Lean Percentage DEXA} (\%)$

2.7 Statistical analyses

A 4 × 2 factorial arrangement of treatments was used to investigate the response of male broiler chickens challenged or not to four ratios of dig Met + Cys:dig Lys in the diets. Data were analyzed using the GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The cage served as the EU for growth performance, and one bird served as the EU for determining mRNA content, relative weights of liver and spleen, and DEXA estimated fat and lean mass. The differences were considered significant

at an alpha level of 0.05. A linear regression model was used to evaluate the effects of the dig Met + Cys:dig Lys ratio on the evaluated parameters.

3. RESULTS AND DISCUSSION

No mortality was found during the experiment. There was no interaction ($P > 0.05$) between the inflammatory challenge and the increase of dig Met + Cys:dig Lys ratio for WG, FI, and FCR. The different ratios of dig Met + Cys:dig Lys had no significant effect ($P > 0.05$) on the same data observed. However, there were significant differences ($P < 0.05$) between challenged and unchallenged animals. Chickens that received the intraperitoneal application of LPS all had lower performance parameters than those that received a SS application (Table 4).

Table 4. Growth performance of broiler chickens from 14 to 21 days of age.

	Control					Challenged					P-value			
	0.69	0.74	0.79	0.84	Mean	0.69	0.74	0.79	0.84	Mean	SEM ²	Rel	LPS	Rel x LPS
¹ WG	0.620	0.628	0.638	0.635	0.630	0.596	0.575	0.576	0.600	0.586	0.004	0.530	<0.001	0.182
¹ FI	0.782	0.785	0.785	0.781	0.783	0.763	0.748	0.738	0.756	0.751	0.004	0.079	<0.001	0.885
¹ FCR	1.26	1.25	1.23	1.23	1.24	1.28	1.30	1.28	1.26	1.28	0.005	0.190	<0.001	0.472

¹WG: weight gain (kg/Bird); FI: feed intake (kg/Bird); FCR: feed conversion ratio (kg of gain/kg of feed).

²Standard error of the mean.

There was no interaction ($P > 0.05$) between the inflammatory challenge and the increase of the dig Met + Cys:dig Lys ratio regarding the relative weight of the liver and spleen. The different ratios of dig Met + Cys:dig Lys had no significant effect ($P > 0.05$) on the same indices observed. However, there was a significant difference ($P < 0.05$) between challenged and non-challenged animals. The chickens that received an intraperitoneal application of LPS had a heavier liver and spleen than those that received the application of SS (Table 5).

Table 5. Relative organ weights of broiler chickens at 21 days of age.

	Control					Challenged					P-value			Rel x LPS
	0.69	0.74	0.79	0.84	Mean	0.69	0.74	0.79	0.84	Mean	SEM ¹	Rel	LPS	
Liver	2.384	2.451	2.369	2.376	2.395	2.468	2.815	2.574	2.623	2.620	0.043	0.324	0.008	0.685
Spleen	0.090	0.094	0.086	0.087	0.089	0.127	0.125	0.124	0.128	0.126	0.004	0.987	<0.001	0.973

¹Standard error of the mean.

There was no interaction ($P > 0.05$) between the inflammatory challenge and the increase in the dig Met + Cys:dig Lys ratio for mRNA content of NF-Kb, GPX, SOD, GSS, MAT2, and CBS in the jejunum. Further, the different ratios of dig Met + Cys:dig Lys had no significant effect ($P > 0.05$) on mRNA content on the same target genes. However, there was a significant difference ($P < 0.05$) between challenged and non-challenged animals for GSS mRNA content. Broilers that received an intraperitoneal application of LPS had lower expression than those that received SS (Table 6).

Table 6. mRNA expression of NF-κB, GPX, SOD, GSS, MAT, and CBS in the jejunum of broilers at 21 days of age.

	Control					Challenged					P-value			
	0.69	0.74	0.79	0.84	Mean	0.69	0.74	0.79	0.84	Mean	SEM ²	Rel	LPS	Rel x LPS
NF-Kb ¹	1.583	1.667	1.717	1.548	1.629	1.260	1.434	1.717	1.548	1.490	0.115	0.790	0.770	0.844
GPX ¹	1.267	1.297	1.849	1.236	1.412	1.292	0.974	1.157	1.105	1.132	0.105	0.638	0.198	0.686
SOD ¹	1.613	1.719	1.981	1.613	1.732	1.437	1.584	1.787	1.621	1.607	0.087	0.550	0.496	0.978
GSS ¹	0.938	0.588	0.855	0.320	0.675	0.091	-0.082	0.747	-0.023	0.183	0.121	0.219	0.042	0.693
MAT2 ¹	1.599	1.928	2.075	1.503	1.776	1.288	1.411	1.735	1.537	1.493	0.096	0.363	0.148	0.780
CBS ¹	1.749	1.597	1.887	1.082	1.579	1.105	1.854	1.288	1.394	1.410	0.091	0.238	0.341	0.095

¹NF-Kb = nuclear factor kappa-B; GPX = glutathione peroxidase; SOD = superoxide dismutase; GSS = glutathione synthetase; MAT2 = methionine adenosyltransferase 2 beta; CBS = cystathionine β-synthase.

²Standard error of the mean.

There was no interaction ($P > 0.05$) between the inflammatory challenge and the increase of dig Met + Cys: dig Lys ratio for lean and fat mass. The different ratios of dig Met + Cys: dig Lys also had no significant effect ($P > 0.05$) on the same body compositions. Furthermore, there were no significant differences ($P > 0.05$) between challenged and non-challenged birds for DEXA estimated fat and lean mass (Table 7).

Table 7. DEXA estimated fat mass (%) and lean mass (%).

	Control (%)					Challenged (%)					P-value			
	0.69	0.74	0.79	0.84	Mean	0.69	0.74	0.79	0.84	Mean	SEM ¹	Rel	LPS	Rel x LPS
Fat mass	7.620	8.623	8.973	9.600	8.704	8.955	8.988	9.227	9.207	9.094	0.152	0.051	0.179	0.213
Lean mass	91.712	90.571	90.168	89.576	90.507	90.240	90.205	89.968	90.376	90.197	0.162	0.101	0.317	0.087

¹Standard error of the mean.

The application of LPS is commonly used to stimulate the inflammatory response of broilers experimentally (Chen et al., 2020; Kreuz et al., 2020; Qiu et al., 2022). This was again confirmed by this study's results: worsening performance, lymphoid organ hypertrophy, and mRNA content of GSS. In addition, the transsulfuration of Met into Cys increases under challenge conditions, and the amount of Met will be deficient for production. Thus, less Met will be acquired and retained in tissues linked to growth performance (Conde-Aguilera et al., 2013), potentially impacting the quality of prime meats, such as breast muscle (Conde-Aguilera et al., 2016).

The impact on lower performance can be attributed to LPS-induced inflammation that deteriorates the intestinal barrier function (Wang et al., 2022a) and absorption and route change of nutrients to attend homeostatic activities compensating the deficiency (Korver & Klasing, 1997; Zhang et al., 2013). The compromise in the use of Aas by the animal (Li et al., 2007; Liu et al., 2015) alters the nutritional requirement of these nutrients. In this study, it was expected that changing the optimal dig Met + Cys: dig Lys ratio in broilers

submitted or not to inflammatory challenge would improve growth performance. However, this hypothesis was not confirmed, possibly because the level of these Aas in the basal diet was suboptimal, as found by Savaram et al. (2022).

Metabolites of Aas can regulate OS and anti-inflammatory activity (Del Vesco et al., 2015; Zhang et al., 2020). This is justified by the increase in liver and spleen hypertrophy with incremental levels of dietary Aas by the rise in protein biosynthesis and liver activity (Jahanian & Khalifeh-Gholi, 2018) and by the associated enhanced immune cell replication and proliferation (Barekattain et al., 2019). So, one strategy to mitigate the adverse effects of bacterial LPS-induced inflammation is to increase the intake of dig Aas in the diet by increasing the dig Aas:dig Lys ratio (Star et al., 2012; Abdaljaleel et al., 2018; Lisnahan et al., 2022) as Met + Cys. However, results confirm that altering the ideal dig Met + Cys:dig Lys ratio does not reduce oxidative and inflammatory stress by altering the relative weight of the liver and spleen. Nonetheless, animals that received the application of LPS had the highest relative weight of liver and spleen than the non-challenged animals (SS application), which agrees with Lieboldt et al. (2016) and Wang et al. (2022b).

LPS can increase NF-Kb production, which stimulates the expression of inflammatory genes (Yang et al., 2019; Liu et al., 2022). Adding high levels of Met to feed can strengthen the antioxidant system by increasing SOD activity in the proximal intestine (Wang et al., 2019), protecting and repairing cells (Roushdy et al., 2018). Powerful negative radicals are formed in tissues through metabolism or cellular reactions, such as superoxide or singlet oxygen. The SOD enzyme can catalytically convert these free radicals into molecular oxygen (O₂) and hydrogen peroxide (H₂O₂), which is less harmful. However, the exaggerated accumulation of H₂O₂ is toxic to tissues or cells (Ighodaro & Akinloye, 2017).

Met is a precursor of GSH (Gasparino et al., 2017), and its supplementation increases GPX expression (Del Vesco et al., 2015; Zhang et al., 2019b). It plays a vital role in antioxidant defense, indirectly breaking down H₂O₂ to water, using H₂O₂ as a substrate for GSH oxidation (Ighodaro & Akinloye, 2017), and stopping lipid peroxidation (Chen et al., 2017). Thus, GPX contributes to the protection of all cells that utilize oxidative metabolism by eliminating increased reactive species during the inflammatory process. In this study, it was expected that altering the optimal dig Met + Cys:dig Lys ratio for broilers subjected to inflammatory challenge or not would improve

mRNA content of NF-Kb, GPX, and SOD in the jejunum. However, this hypothesis was not confirmed.

According to Li et al. (2017), SOD activity can be improved by S-adenosylmethionine, a vital methyl donor. It is synthesized from Met and adenosine triphosphate through the upregulation of MAT2 (Faraji et al., 2018). S-adenosylmethionine acts in methylation and biosynthesis processes of molecules that increase energy availability for protein deposition, such as the methylation of guanidinoacetic acid to form creatine (Mousavi et al., 2013). This study hypothesized that altering the optimal dig Met + Cys:dig Lys ratio for broilers subjected or not to inflammatory challenge would improve the mRNA content of MAT2 in the jejunum. However, this was not confirmed.

Reverse transsulfuration is a Cys biosynthetic pathway characterized by the cleavage of cystathionine. It starts with CBS separating the complex of homocysteine (HCy) and serine to form cystathionine (Kruger, 2017). In addition to this role, HCy can regenerate Met via remethylation (Wan et al., 2017). This study expected that altering the optimal dig Met + Cys:dig Lys ratio for broilers subjected or not to inflammatory challenge would improve CBS's mRNA content in the jejunum. However, this hypothesis was not confirmed.

GSS is an enzyme that acts in the second step of the formation of GSH by catalyzing the reaction of dipeptide gamma-glutamylcysteine with glycine (Lu, 2013). GSH is present in the intestinal epithelium, where it can improve intestinal morphology characteristics (Song et al., 2018). However, when chickens go through LPS-induced inflammation, there is a lower production of GSH (Sun et al., 2014). Because it depends on the GSS enzyme activity for their formation, it can be stated that there was a reduction in the expression of GSS as well. This study predicted that the alteration of the ideal dig Met + Cys:dig Lys ratio for broilers submitted or not to inflammatory challenge would improve the mRNA content of GSS. However, this hypothesis was not confirmed. On the other hand, findings show that broilers that received LPS-induced inflammation had lower mRNA content of GSS in the jejunum.

SAA stands out in the protein and lipid metabolism of poultry (Majdeddin et al., 2019). This Aas participates in pathways that give rise to polyamines, creatine, melatonin, and epinephrine (Zhou et al., 2020). In addition, SAA participates in metabolic pathways of methyl group donation, such as choline and betaine (Alagawany et al., 2022). Regarding lipid metabolism, SAA interferes with the oxidative catabolism of fat tissue (Elsharkawy et al., 2021), like carnitine biosynthesis (Ringseis et al., 2018) that

stimulates fatty acid oxidation for better energy production, growth performance (Golrokh et al., 2016), and improves carcass quality, as reported by Asadi et al. (2016). Thus, this study hypothesized that altering the ideal dig Met + Cys:dig Lys ratio for broilers submitted or not to inflammatory challenge would change the percentage of lean and fat mass. However, results revealed that these variables were not influenced.

4. CONCLUSION

The lowest dig Met + Cys:dig Lys ratio, that is 0.69 (1.193% dig Lys: 0.823% dig Met + Cys), was sufficient in maintaining the performance parameters, the relative weight of lymphoid organs, fat and lean mass, and NF-Kb, GPX, SOD, GSS, MAT2, and CBS mRNA content of jejunum.

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UNIVERSIDADE FEDERAL DE VIÇOSA
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Viçosa, 17 de Dezembro de 2021

CERTIFICADO

Certificamos que o projeto intitulado "Relação ideal metionina + cisteína: lisina em dietas para frangos de corte submetidos desafio inflamatório", protocolo nº 070/2021, sob a responsabilidade de Arele Arlindo Calderano - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de 16 de Dezembro de 2021.

Finalidade: () Pesquisa () Ensino Vigência do Projeto: de 01 de fevereiro de 2022 a 30 de Set. 2022
 Espécie/linhagem: Frango de corte (*Gallus gallus domesticus*) Nº de animais: 384
 Peso: 0,430Kg Idade: 13 dias Sexo: Macho Origem: Incubatorio Rivelli Cnpj/CPF: 478.715.616-49
 Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecilia CRMV: 10595

CERTIFICATE

We certify that the project entitled "Ideal methionine + cysteine: lysine ratio in diets for broilers submitted to inflammatory challenge", protocol nº 070/2021, under the responsibility of Arele Arlindo Calderano - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on Dec. 16th of 2021.

Finality: () Research () Education
 Duration of the Project: from Feb. 1st of 2022 to Sep. 30th, 2022.
 Species / strain: Broiler (*Gallus gallus domesticus*) Nº of animals: 384
 Weight: 0,430Kg Age: 13 days Sex: Male Source: Incubatorio Rivelli Cnpj/CPF: 478.715.616-49
 Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecilia CRMV: 10595

Luciana Navajas Rennó

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 Coordenadora da CEUAP/UFV